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4. **Title of the invention**

Biological indicator

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BIOLOGICAL INDICATOR

The invention relates to a biological indicator, based on enzymes, suitable for the validation of processes, including heat-based inactivation processes and inactivation procedures in general and specifically for the validation of procedures to inactivate transmissible spongiform encephalopathy (TSE) agents.

Creutzfeldt-Jakob Disease (CJD) is a relatively rare form of human neurodegenerative disorder presenting as either a familial, sporadic or iatrogenic disease at a frequency of approximately 1 case per million population. The emergence of a new variant form (vCJD) of the disease predominantly in a younger age group, possibly due to consumption of bovine spongiform encephalopathy (BSE)-infected meat products; has raised the possibility of a large increase in the numbers of cases. These factors have important public health consequences. A large proportion of the UK population has potentially been exposed to the disease via food during the late 1980s. Whilst the number of cases to date has been relatively low (149 cases to February 2004) there remains a significant risk, from all forms of CJD, via other transmission routes including surgery, transplants, transfusion or contaminated medical products. A number of these routes have been implicated in the iatrogenic spread of the disease in a clinical setting and others have been defined in animal models.

The agents responsible for causing all forms of CJD in humans are highly resistant to inactivation by standard methods. Validated methods for the decontamination of surgical instruments are urgently required. A variety of treatments, including chemical treatments and the use of high temperatures and pressures with wet or dry heat, have been tested but none are adequate [Taylor, D.M. (1999) in: Principles and practice of disinfection, preservation and sterilisation. (Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J., Eds): pp 222-236 Blackwell Scientific Publications, Oxford; Taylor, D.M.. (2001) Contrib Microbiol. 7, 58-67; Taylor, D.M., Fernie, K, Steele, P.J., McConnell, I. and Somerville, R.A.. (2002) J Gen Virol. 83, 3199-3294]. Incineration is effective, but precludes any recovery or reuse of raw materials or equipment. The use of high concentrations of sodium hydroxide (up to 2M) or high levels of sodium hypochlorite (up to 20000ppm) have been shown to significantly reduce the levels of TSE agents, but

have a deleterious effect on surgical instruments and may be harmful to the operator. A wide variety of other methods have been proposed as means of inactivating TSE agents on surgical instruments and are currently in development. These include a variety of gaseous phase sterilants including vapour phase hydrogen peroxide, ozone and ethylene oxide. Other methods have been proposed as a specific anti-TSE pre-treatment prior to routine sterilisation and these include treatment of the surgical instruments using thermostable proteases under defined conditions of pH and temperature.

Inactivation of *Bacillus stearothermophilus* spores is a method routinely used to validate the correct performance of autoclaves. Such indicators may represent a relevant indication that a bacteria or virus has been inactivated by the process, with the process usually validated as reducing the level of infectious agent by an order of 10^6. However, the uniquely stable properties of the TSE agents mean that a much more robust indicator is required to provide a relevant indication of the performance of processes to inactivate such agents.

Other biological indicators, based on thermostable spores or enzymatic preparations are well known to those familiar with the art. All have the drawback that they are unable to validate inactivation of an infectious agent in excess of a 10^6 reduction in activity.

Inactivation of TSE agents is also a significant issue for the disposal of Bovine Spongiform Encephalopathy (BSE)-infectious materials and in the preparation of raw materials of animal origin. There is now a good body of scientific evidence that the emergence and spread of BSE was via changes in rendering practice with highly infectious neuronal tissue being fed back to cattle via meat-and-bone-meal supplements. There is also good evidence that BSE was the cause of vCJD, almost certainly as the result of eating contaminated beef products. For this reason any cattle that die of BSE and spinal cord and brain tissue from all cattle are currently removed from the food chain and disposed of by an alternative route. This has the result that enormous amounts of animal waste is currently being accumulated or disposed of by incineration. The treatment of such material with thermostable proteases is one possible solution. Again there is a requirement for a validation procedure to ensure that any infectious material is destroyed in an appropriate process.

It is an object of the present invention to provide an alternative and/or improved biological indicator and uses thereof.

Accordingly, the present invention provides a composition, comprising:-

(1) a kinase, and

(2) a solid support,

wherein the kinase is immobilised in or is immobilised on the solid support.

The composition is advantageously used as a biological indicator. As an example, a biological indicator is included in a sample that is being treated to reduce its content of a potential contaminant, especially an infectious agent. It is known from previous tests that the activity of the indicator will be reduced by the treatment at a rate that can be correlated with reduction in activity of the contaminant. To determine whether the level of the contaminant has been reduced below an acceptable level, the amount of the indicator is measured before and after, or during, the treatment. When a level of indicator is reached that is known to correlate with an acceptable reduction in the contaminant the treatment is then regarded as validated, and if the contaminant is an infectious agent then the sample may be regarded as sterile.

In a use of the invention, thermostable kinase is the reporter in a method of indicating the possible presence of infectious TSE agents following a cleaning or inactivation procedure. First, a sample containing thermostable adenylate, acetate or pyruvate kinase is exposed to a cleaning/inactivation procedure. The next step is to remove any contaminating enzymatic activity by heat treatment, e.g., at from 60 to 80^{0}C for at least 10 minutes. The thermostable enzyme is then reacted with a substrate to allow the generation of ATP, at a temperature of between 30°C and 70°C, which can be measured by bioluminescent detection using luciferin / luciferase and a suitable luminometer at 20-30^{0}C for 10 minutes to 1 hour. Based on previous data, the method is completed by correlating the level of remaining enzymatic activity with the possible presence of an infectious TSE agent within the treated sample.

Kinase enzymes have been found to be capable of generating a signal that is detectable over an extremely wide range. Generally, the kinase is detected using a substrate comprising ADP which is converted to ATP, itself used to generate

light, eg. using luciferin/luciferase, detected using a luminometer. The wide range makes the indicator particularly suitable for validation as the kinase remains detectable even after many logs reduction in activity. For sterility, most national institutes regard a 6 log reduction in activity as required before sterility can be validated. The kinases of the invention offer the potential of validating reduction in activity of infectious agents well beyond 6 logs, to 8 logs and more, thus increasing the scope of monitoring offered at present.

In preferred embodiments of the invention, the kinase is thermostable, and is hence suitable for use in validation of processes carried out at high temperature. Thermostable kinases are also found to be resistant to other extreme environments, and are for example often found resistant to extremes of pH and resistant to exposure to proteolytic enzymes. So the kinases of the invention can be used for monitoring treatments of infectious agents that employ one or a combination or all of high pH, high temperature and proteases.

By thermostable is meant that at least 95% of the activity of the kinase is retained after exposure to 70 degrees C for 30 minutes. Preferred enzymes of the invention are very thermostable and will retain at least 95% activity after heating to 80 degrees C for 10 minutes. The kinases from mesophilic organisms and even a variety of thermophilic organisms, such as *Bacillus stearothermophilus* (used widely as a biological indicator) do not meet these criteria, but may nevertheless be suitable as indicators for inactivation carried out at lower temperatures.

The kinases used in particular embodiments of the invention are adenylate kinase, acetate kinase and pyruvate kinase, or combinations thereof. Further, the adenylate, acetate and pyruvate kinase enzymes may be obtained from *Pyrococcus furiousus*, *P.abyssi*, *P.horikoshii*, *P.woesii*, *Sulfolobus solfataricus*, *S.acidocaldarius*, *S.shibatae*, *Rhodothermus marinus*, *Thermococcus litoralis*, *Thermatoga maritima*, *Thermatoga neapolitana* and *Methanococcus* spp. Adenylate kinase is especially preferred and has been used in examples of the invention set out in detail below. The kinases catalyse formation of ATP from a substrate comprising ADP, and the ATP is then readily detected using known methods and reagents. Specific kinases suitable for the invention are set out in SEQ ID NO.s 1-19.

The biological indicator can be adapted for use in a vessel in which an infected sample (or a sample believed to be infected) is being processed. For example, the indicator includes a solid support which is a matrix and the kinase is disbursed within the matrix. The matrix should be resistant to the process conditions and can help to provide protection to the kinase from the process – hence providing some stabilization to the indicator. The kinase can be located within a polymer matrix. The support can be designed to project into or be added to a processing vessel, and can be an indicator strip, a dip stick or a bead.

A variety of solid supports, with or without chemical modifications and with one or more kinase indicators in a variety of formulations, are embraced by the invention, depending e.g. on the requirements of the process to be validated. In one form the support is a plastic, ceramic, steel or other metallic or polymer surface onto which the kinase is dried as a means of immobilisation. The support can be a polycarbonate, polystyrene or polypropylene strip or dipstick, possibly with a flattened surface, onto which the kinase is applied. An additional type of support with a porous surface for attachment of kinase is also particularly useful as an indicator for gaseous inactivation methods. Kinase coated plastic, metallic or ceramic beads may also provide a valuable format for the indicator, again with specific relevance to monitoring gaseous processes. Such supports have advantages over solid supports for certain applications, as they provide a significantly increased surface area for the attachment of the indicator.

For the specific example of a device formulated for the validation of TSE inactivation processes, a steel surface such as a rod, disk or coupon is an effective indicator for surgical instrument decontamination. The binding of a thermostable adenylate kinase, for example, is a very good model of the aggregated form of the disease-related prion isoform (PrP^{Sc}) and as such provides a remarkably good indicator of PrP^{Sc}'s interaction with the surface of surgical steel.

Binding of kinase on or to the support of the indicator may be achieved by methods routinely used to link protein to surfaces, e.g. incubation of protein in 0.1M sodium bicarbonate buffer at about pH9.6 at room temperature for about 1 hour. Alternatively the protein is covalently coupled to the surface using any of a wide range of coupling chemistries known to those familiar with the art. For example an adenylate kinase derivitised with SPDP (Pierce chemicals; using

manufacturer's instructions), reduced with DTT to provide free sulfhydryl groups for cross-linking, is covalently attached to a polystyrene support with a maleimide surface. Plastic surfaces with such sulfhydryl-binding surfaces are well described in the literature. An added benefit of this method of coupling is that, if required, the enzyme can be cleaved from the support eg. by reduction with DTT or MESNA, to allow the assay to be carried out separately to any indicator support. The adenylate kinase enzymes and other indicator kinases described by this invention have the property that their activity is retained upon derivitisation and cross-linking to such supports. Alternatively an amine reactive surface on a polystyrene or polycarbonate support is used, with a bifunctional cross-linking agent such as monomeric glutaraldehyde, to provide direct non-cleavable cross-linking of the kinase via free amine groups on the protein. UV treatment can also be used to directly link the indicator to a suitable support. Steel surfaces can be treated in a similar way to plastic surfaces to mediate covalent attachment of the indicator kinase.

For each type of support the kinase is preferably formulated in a solution that enhances binding and/or stabilises the bound protein. Such formulations include solutions containing up to 10% (w/v) sucrose, sorbitol, mannitol, cellulose, or polyethylene glycol (PEG). In addition the kinase can be formulated as part of a gel that is applied to the surface or lumen of a suitable support. Examples include alginate, agar or polyacrylamide matrices.

The composition may also comprise an agent to stabilise the kinase, and suitable stabilising agents are selected from metal ions, sugars, sugar alcohols and gel-forming agents.

To facilitate use of the indicator, it may further comprise means to attach the composition to a surface, such as a projection, recess or aperture for attachment of the composition to a surface by means of a screw, nut and bolt or clamp.

In specific embodiments of the invention, purified adenylate kinase (AK) is formulated at a concentration of up to 1mg/ml and coated onto solid supports. For protease treatment, the AK is dried onto a polypropylene, polycarbonate or polystyrene surface similar to microtitre plates. For either standard autoclaving at 121°C for 15-20 minutes or "prion-cycle" autoclaving at 134°C for 18 minutes, a

heat-stable support such as stainless steel is used. For gas phase inactivation procedures such as hydrogen peroxide or ozone, polycarbonate solid support is used, and can also be manufactured as a porous matrix to provide a greater degree of resistance to the inactivant if required.

A convenient solid support takes the form of a dipstick which is transferred directly from the inactivation procedure to a tube containing all the required assay components. This can be a tube luminometer attached to one of a range of "rapid read-out" hygiene monitors already on the market for the food and pharmaceutical industry. Alternatively it can take the form of a specialised instrument designed for the indicator in question, with a particular emphasis on maintaining the optimal temperatures required by the thermostable enzymes.

The present invention also provides a biological indicator comprising a plurality of enzymes detectable after differing levels of inactivation. A biological indicator of one embodiment comprises a support, a first enzyme located at a first position and a second enzyme located at a second position separate from the first position, wherein both enzymes have activity in converting product to substrate and after exposure of the biological indicator to an inactivation process for an initial period of time activity of both enzymes can be detected, after exposure of the biological indicator to the inactivation process for a subsequent period of time activity of the first enzyme cannot be detected but activity of the second enzyme can be detected, and after exposure of the biological indicator to the inactivation process for a second subsequent period of time activity of the second enzyme cannot be detected.

An advantage of this embodiment is that the indicator can be used to show an approximate level of inactivation achieved by the process without the need for a precise measurement to be taken. Thus, for example, when both enzymes are detectable this can indicate that the inactivation has not reached a certain threshold. When only the second enzyme can be detected this indicates that the first threshold of inactivation has been reached but a second threshold has not, and lastly when neither enzyme can any longer be detected this indicates that the inactivation has passed the second threshold. If the first enzyme is detectable at up to 6 logs reduction inactivity and the second enzyme is detectable at up to 8 logs reduction inactivity then being able to detect both enzymes indicates that inactivation has not reached 6 logs, being able to detect only the second enzyme indicates inactivity has

been reduced by between 6 and 8 logs and being able to detect neither indicates that at least 8 logs reduction in activity has been achieved.

The first enzyme is suitably detectable at up to between 5 and 8 logs reduction in activity and the second enzyme is suitably detectable at 6 logs or greater reduction in activity, the first enzyme preferably detectable at between 6 and 7 logs reduction in activity and the second enzyme being detectable at between 7 and 8 logs reduction in activity. The biological indicator may further comprise a third enzyme located at a third position separate from the first and second positions, wherein after exposure of the biological indicator to the inactivation process for the second subsequent period of time the third enzyme can be detected and after exposure of the biological indicator to the inactivation process for a third subsequent period of time the third enzyme cannot be detected. The third enzyme is suitably detectable at 8 logs reduction in activity or greater.

Using a biological indicator of this type, having multiple enzymes which are detectable at differing levels of activity, the progression of the inactivation progress can be watched and its end point anticipated readily.

The preferred enzymes of the multi-enzyme indicator are kinases, more preferably thermostable and more preferably as disclosed and described herein in relation to other embodiments of the invention.

The invention also provides a kit for determining whether an infectious agent has been inactivated, comprising:-

(i) a composition of the invention, and

(ii) substrate for the kinase.

To carry out measurement of the kinase amount / activity, the kit can include means for detecting ATP, e.g. luciferin/luciferase and optionally a luminometer.

From previous testing with known infectious agents, data correlating inactivation with reduction in kinase activity can be prepared, and the kit therefore can also include one or more look-up tables correlating kinase inactivation with inactivation of a list of specified infectious agents.

The invention is for use in validating inactivation of a wide range of infectious agents, including bacteria, viruses, spores and proteins. It is particularly suited for validation of inactivation of infectious agents that retain infectivity after 7 or greater log reduction in activity, and especially for methods of inactivation of TSE.

In a further aspect of the invention there is provided a method of determining the extent of inactivation of an infectious agent, comprising:-

(i) obtaining a sample containing the infectious agent and a known amount of a kinase;

(ii) treating the sample to inactivate the infectious agent;

(iii) measuring residual kinase; and

(iv) thereby determining residual infectious agent.

This method can be used to measure reduction in the amount or activity of the infectious agents, as the extent of inactivation of the infectious agent correlates with the extent of inactivation of the kinase.

The sample generally is provided without any kinase present, so the method comprises obtaining a sample believed to contain the infectious agent and adding a known amount of kinase. The agent may not be present at all. The point of the validation is that, after carrying out the treatment, it is confirmed that any agent that might have been present has been inactivated to an acceptable degree.

Typically, an operator measures kinase activity prior to treating the sample and after treating the sample. It is also possible that contaminating, usually mesophilic kinase can get into the sample prior to assaying for kinase activity. It is thus preferred that the kinase is thermostable and that the assay step includes inactivating mesophilic kinase, such as by treating the sample at 80 degrees C for at least 10 minutes prior to measuring residual kinase.

The treatment can be substantially any designed to destroy an infectious agent. Suitable treatments include one or more of high pH, high temperature and high pressure, and exposing the sample to a protease.

In a specific embodiment of the invention described below in more detail, the treatment comprises exposing the sample to a thermostable protease at a

temperature of 60 degrees C or above, more particularly exposing the sample to the protease at a pH of 9 or above.

The treatment may also be a cleaning or inactivation process which includes high temperature autoclaving with wet or dry steam, chemical treatments, protease or other enzymatic treatments, ozone sterilisation, H_2O_2 sterilisation, rendering or other method designed to eliminate or inactivate the TSE agents within a sample or on the surface of an instrument or tool.

Still further provided by the invention is a method of correlating inactivation of an infectious agent by a treatment with inactivation of an indicator by the treatment, comprising:-

(i) obtaining a sample containing a known amount of the infectious agent and a known amount of the indicator;

(ii) carrying out the treatment on the sample;

(iii) measuring residual infectious agent; and

(iv) measuring residual indicator.

This enables preparation of data for future use of the indicator for validation of the treatment on samples suspected of containing the infectious agent. We have correlated various treatments using the kinase indicators of the invention as described herein and found them operable at up to and beyond a reduction in activity of 8 logs, useful for monitoring of treatments in general but especially TSE inactivation.

Hence, in using the invention, a particular treatment method comprises:-

adding a biological indicator to the sample;

treating the sample;

assaying for residual activity of the biological indicator; and

continuing the treatment until the biological indicator activity indicates a reduction in activity of transmissible spongiform encephalopathy of 8 logs or greater.

The invention additionally provides use of a thermostable kinase in manufacture of a biological indicator, preferably wherein the biological indicator is for validating inactivation of infectious agents.

ATP bioluminescent detection is a preferred means of detecting kinase activity. A standard luciferin-luciferase assay method can detect as little as 10^{-15} moles of ATP. By coupling an enzymatic amplification to the bioluminescent detection methods it is possible to detect as few as 10^{-20} moles of kinase. This type of format therefore offers remarkable sensitivity for the detection of molecules using binding species linked to adenylate kinase (AK) as described in WO 02/053723.

Use of a kinase, e.g. AK, coupled to bioluminescent detection has a number of other significant advantages. The assay gives a direct relationship between enzymatic activity and light production over a much larger range than other comparable assay formats. Thus whilst an assay using a traditional reporter enzyme such as horseradish peroxidase or alkaline phosphatase will give a proportional response over 5-6 log dilutions, the AK-luciferase assay can provide a dynamic range of at least 8 logs. As direct indicators this makes them especially useful for processes which require a level of inactivation greater than the standard 6-log range as the signal can be made to be meaningful across the whole range of the assay, something that would not be possible using other assay formats. This is particularly relevant for TSE inactivation where, in a worse case scenario, as many as 8-logs of infectivity may be present on the surface of a surgical instrument, assuming the presence of 1mg brain tissue at a level of up to 10^8 TSE infectious units per mg. Under these circumstances an indicator of the invention, providing an 8-log range of signal is particularly valuable.

Given the type of processes for which a TSE indicator is required a high level of both thermal and physical stability is preferred. In an example below, the properties of a range of AK enzymes from thermophilic organisms were compared. Even AKs from thermophilic organisms such as the indicator strain *B.stearothermophilus* lose the majority of their activity at relatively low temperatures. For a kinase-based indicator to be included in an autoclaving cycle a significantly greater degree of thermostability, such as that demonstrated by the enzymes from the *Sulfolobus* species or *Pyrococcus furiosus,* is used.

A number of additives and changes to formulation that increase the stability of an immobilised enzyme to heat inactivation will be known to those familiar with the art. The thermostable enzymes used in embodiments of this invention will require significantly less stabilisation given that they are already significantly more stable than other enzymes used for this type of process. AK enzymes described herein, in particular the AK enzymes from *Sulfolobus acidocaldarius*, *S solfataricus*, *S.shibatae*, *Pyrococcus furiosus*, *Rhodothermus marinus* and *Thermococcus litoralis*, are significantly more stable at both 80°C and 90°C than even the enzyme from an organism normally used as an indicator of process sterilisation such as *Bacillus stearothermophilus*. In many cases these AK enzymes immobilised on a solid support may require no further stabilisation to provide the necessary range of activity to be measured following autoclaving, pasteurisation or equivalent.

The addition of stabilising agents such as sorbitol up to a concentration of 4M, or other polyols such as ethylene glycol, glycerol, or mannitol at a concentration of up to 2M may improve the thermostability of the enzyme. Other additives such as xylan, trehalose, gelatin may also provide additional stabilisation effects either individually or in combination. Addition of a range of divalent metal ions, most notably Ca^{2+}, Mg^{2+} or Mn^{2+} may also improve stability of the enzyme.

Chemical modification of the AK enzymes can also be used to improve their thermal stability. Reductive alkylation of surface exposed amino groups by glyoxylic acid (e.g Melik-Nubarov (1987) *Biotech letts* 9:725-730), addition of carbohydrates to the protein surface (e.g. Klibanov (1979) *Anal. Biochem.* 93:1-25) and amidation (e.g. Klibanov (1983) *Adv. Appl. Microbiol.* 29:1-28) may all increase the stability of the enzyme. Further methods including the use of chemical cross-linking agents and the use of various polymeric supports for enzyme immobilisation are also relevant methods for increasing the thermostability of AK enzymes (reviewed in Gupta (1991) Biotech. Appl. Biochem. 14:1-11).

Similar modifications are also relevant to the stabilisation of the AK indicator against other sterilisation processes such as hydrogen peroxide or ozone. In particular, processes where the access of the gaseous phase sterilant to the enzyme is restricted, for example by encapsulation in a suitable polymer or

formulation with an additive to reduce penetration of the gas, will provide useful methods for increasing the stability of the enzyme if required.

Many of the treatments that are effective at increasing the thermal stability of enzymes are also relevant to the stabilisation for protease treatments, e.g. for the development of an indicator for the effective inactivation of TSE agents by protease treatment. In general a protein that shows high levels of thermostability is likely to also show a high degree of stability for degradative processes such as denaturation or protease treatment (See for example: Daniel RM, Cowan DA, Morgan HW, Curran MP, "A correlation between protein thermostability and resistance to proteolysis", Biochem J. 1982 207:641-4; Rees DC, Robertson AD, "Some thermodynamic implications for the thermostability of proteins", Protein Sci. 2001 10:1187-94; Burdette DS, Tchernajencko V V, Zeikus JG."Effect of thermal and chemical denaturants on Thermoanaerobacter ethanolicus secondary-alcohol dehydrogenase stability and activity", Enzyme Microb Technol. 2000 27:11-18; Scandurra R, Consalvi V, Chiaraluce R, Politi L, Engel PC., "Protein thermostability in extremophiles", Biochimie. 1998 Nov;80(11):933-41; and Liao HH., "Thermostable mutants of kanamycin nucleotidyltransferase are also more stable to proteinase K, urea, detergents, and water-miscible organic solvents", Enzyme Microb Technol. 1993 Apr;15(4):286-92). The thermostable adenylate kinases therefore generally show a higher degree of stability to the actions of the protease treatments designed to inactivate TSE agents than might an equivalent mesophilic AK. Depending on the type of process used, an AK can also be selected to favour other characteristics of the process. Thus for a protease treatment at alkaline pH the protocol tends towards the use of a thermostable AK from a moderately alkalophilic organism such as *P.furiosus*, whereas a protease treatment at acidic pH might use an AK from an acidophile such as *S.acidocaldarius* or *S.solfotaricus*.

If required to improve the stability of the AK indicator to protease treatment a number of other options exist. A number of these are the same as those described above for the stabilisation of the enzyme against heat treatment. For example formulations containing sorbitol, mannitol or other complex polymers reduce the levels of inactivation of the AK enzyme on the indicator surface. In addition treatments that specifically reduce the rate at which a protease substrate is degraded are particularly relevant to this application. For example the

formulation of the AK in a solution containing up to around 10mg/ml (a 10-fold excess compared to the maximal concentration of the indicator) of a suitable carrier protein such as casein or albumin, that acts as alternative substrate for the protease, will specifically reduce the rate of digestion of the AK indicator. Similarly the addition of free amino acids such as glycine, tyrosine, tryptophan or dipeptides to the formulation would provide a means of substrate level inhibition of the enzyme and reduce local inactivation of the AK indicator.

The invention additionally provides methods for the production of various thermostable adenylate kinases by recombinant expression in bacteria for use in manufacture of biological indicators.

The genetic modification of enzymes has been shown to provide significant increases in thermal stability and by analogy such mutations are also likely to significantly enhance the stability of the AK indicator enzymes in other processes such as protease treatment or gaseous phase "sterilisation". The comparison of the thermostability of the AK enzymes shown in Figure 1 taken with the defined 3-D structure of the trimeric (archaeal) AKs (Vonrhein *et al* (1998) *J. Mol. Biol.* **282**:167-179 and Criswell *et al* (2003) *J. Mol. Biol.***330**:1087-1099) has identified amino acids that influence the stability of the enzyme.

Genetically engineered variants of kinases showing improved thermostability are also provided by and used in the invention, and can be generated in a number of ways. Essentially these involve the specific site-directed mutagenesis of amino acids believed to form part of the central core packing region of the trimeric molecule and random "directed evolution" methods where the whole molecule is subjected to subsequent rounds of mutagenesis and selection/screening of molecules with improved properties. The modifications outlined herein, e.g. in an example below, are based on a hybrid approach using a consensus based approach to define regions likely to influence the thermostability of the enzymes based on observed differences between structurally related molecules. This is followed by either defined changes to incorporate the amino acids that correlate with the best thermostability or a random replacement to incorporate every available amino acid at the positions defined as being essential for thermostability.

Specific modified enzymes of the invention are the various variants set out in Examples 6-8 and referred to collectively as SEQ ID NO.s 15-17 (though several variants are embraced by each reference).

The invention is now described in specific embodiments in the following examples and with reference to the accompanying drawings in which:-

Figure 1 shows activity of adenylate kinase enzymes after treatment at 70°C (A), 80°C (B) and 90°C (C);

Figure 2 shows residual enzyme activity after digestion of adenylate kinase with differing concentrations of alkaline protease; and

Figure 3 shows standard curves correlating enzyme activity with residual concentration of adenylate kinase.

Example 1

Assay Protocol for *Sulfolobus acidocaldarius* AK

Adenylate kinase formulated at a concentration of 1mg/ml on a suitable solid support is the indicator and is subjected to the inactivation process as required.

A heat inactivation step is optional. This involves heating the sample to a temperature permissive for the indicator AK but above that at which any mesophilic AKs are denatured. Typically this is by incubation at 80°C for 10 minutes.

A washing step is optional and may be incorporated to remove any trace of "inactivant" if this is found to interfere with the assay.

The indicator is then added to a tube containing ADP substrate (e.g. Celsis) at a concentration of 13.5µM diluted in 15mM MgAc, 1mM EDTA buffer, pH 6.8 and incubated at 70°C for 20 minutes.

The sample is cooled to room temperature and luciferin/luciferase substrate (ATP Reagent, Thermo Life Science) added. The assay is incubated for the required length of time and a luminometer is used to read the sample.

Based on a titration curve of enzyme activity and infectious agent activity for the formulated indicator the extent of inactivation of the enzyme, and hence infectious agent, is calculated. Generally, a greater than 6-log reduction in the level of signal is required as a minimum level of inactivation. The process or statutory requirements may dictate other more stringent requirements in terms of signal reduction. These may be defined at the start of the process to allow an operator to continue with further manipulations provided the reading is below a specified threshold level.

Example 2

Analysis of thermostability of native adenylate kinase enzymes.

Biomass was produced from twenty-four diverse thermophilic and hyperthermophilic microorganisms (Table 1). Eight members of the archaea were represented along with sixteen diverse aerobic and anaerobic bacteria. AKs from each of these organisms was purified by affinity chromatography using selective absorption and desorption from Cibacron Blue 3A (Blue Sepharose). All enzymes were further characterised and purified by gel filtration (Superdex G200). This enabled identification of the major AK fraction and estimation of molecular mass. ATP bioluminescence was used to determine the activity of the AKs over a range of temperatures and to define thermal stability.

Example 3

Thermostable adenylate kinases

The thermostability at 70, 80 and 90°C of adenylate kinases isolated from biomass from thermophilic organisms was assessed, and the results shown in Fig. 1. The adenylate kinases were isolated from the biomass by affinity chromatography using selective absorption and desorption from Cibacron Blue 3A (Blue Sepharose). The samples eluted from the columns were diluted 1:10 000 and then

10µl of each added to a microtitre well. 2.5µl of apyrase was added to each well to destroy the ATP present from the elution buffer, and incubated at 37°C for 30 minutes. The apyrase was inactivated by heat treatment at 65°C for 20 minutes. ADP substrate was added and incubated at either 70 (panel A), 80 (panel B) or 90°C (panel C) for 30 minutes and cooled to 25°C before the addition of 10µl of D-luciferin-luciferase reagent. The ATP produced was measured as RLU on a plate luminometer.

Example 4

Expression and purification of thermostable adenylate kinases

Clones expressing representative thermostable AKs were secured and recombinant thermostable AKs from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* and the thermophilic bacterium, *Bacillus stearothermophilus* produced. The plasmids were transformed into *E.coli* and the cell extracts shown to contain protein bands on electrophoresis corresponding to the expected molecular masses of the AKs. Thermostable AK activity was measured after incubation at the appropriate temperature (80°C for the *Sulfolobus acidocaldarius* AK and 60°C for the *Bacillus stearothermophilus* AK). Purification methods for both thermostable AKs were established and included an initial heat treatment of incubation for 20min at 80°C, to inactivate and aggregate proteins derived from *E.coli*, followed by affinity chromatography and gel filtration. The affinity chromatography involved adsorption of the enzyme to Blue Sepharose, followed by specific elution with a low concentration of AK co-factors (AMP+ATP and magnesium ions). The ATP and AMP (Sigma) in the elution buffer were degraded by incubation with mesophile apyrase, which is readily inactivated by subsequent heat treatment. Gel filtration chromatography was scaled up to utilise a preparation grade Superdex column to enable large quantities of both enzymes to be prepared.

Primers were designed for PCR amplification of the AK genes from the thermophilic organisms identified during the screening of candidate native enzymes.

The thermostable microorganisms were grown using individually defined growth conditions and genomic DNA isolated and used as templates for PCR amplification of the adenylate kinase genes from each organism. PCR amplified adenylate kinase genes from the thermophilic organisms, *Thermotoga maritima, Aeropyrum pernix, Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* were sub-cloned into the vector, pET28a and transformed into a codon enhanced *E.coli* strain expressing rare tRNAs (Zdanovsky *et al,* 2000). This *E.coli* strain is suitable for enhancing expression levels of AT-rich genes.

The success of the transformation was assessed by a mini-expression study, and the results analysed by SDS-PAGE of the culture supernatants before and after induction with IPTG. SDS-PAGE was also used to analyse the supernatants after inclusion of a heat treatment step, which consisted of heating the sample to 80°C for 20 minutes prior to running on the SDS-PAGE gel to remove heat labile proteins present in the sample.

Sequences:

Seq IDs

1 - Adenylate kinase from *Sulfolobus solfataricus*

MKIGIVTGIP GVGKTTVLSF ADKILTEKGI SHKIVNYGDY MLNTALKEGY VKSRDEIRKL QIEKQRELQA
LAARRIVEDL SLLGDEGIGL IDTHAVIRTP AGYLPGLPRH VIEVLSPKVI FLLEADPKII LERQKRDSSR
ARTDYSDTAV INEVIQFARY SAMASAVLVG ASVKVVVNQE GDPSIAASEI INSLM

2 - Adenylate kinase from *Sulfolobus acidocaldarius*

MKIGIVTGIP GVGKSTVLAK VKEILDNQGI NNKIINYGDF MLATALKLGY AKDRDEMRKL SVEKQKKLQI
DAAKGIAEEA RAGGEGYLFI DTHAVIRTPS GYLPGLPSYV ITEINPSVIF LLEADPKIIL SRQKRDTTRN
RNDYSDESVI LETINFARYA ATASAVLAGS TVKVIVNVEG DPSIAANEII RSMK

3 - Adenylate kinase from *Sulfolobus tokodaii*

MSKMKIGIVT GIPGVGKTTV LSKVKEILEE KKINNKIVNY GDYMLMTAMK LGYVNNRDEM RKLPVEKQKQ
LQIEAARGIA NEAKEGGDGL LFIDTHAVIR TPSGYLPGLP KYVIEEINPR VIFLLEADPK VILDRQKRDT
SRSRSDYSDE RIISETINFA RYAAMASAVL VGATVKIVIN VEGDPAVAAN EIINSML

4 - Adenylate kinase from *Pyrococcus furiosus*

MPFVVIITGI PGVGKSTITR LALQRTKAKF RLINFGDLMF EEAVKAGLVK HRDEMRKLPL KIQRELQMKA
AKKITEMAKE HPILVDTHAT IKTPHGYMLG LPYEVVKTLN PNFIVIIEAT PSEILGRRLR DLKRDRDVET
EEQIQRHQDL NRAAAIAYAM HSNALIKIIE NHEDKGLEEA VNELVKILDL AVNEYA

5 - Adenylate kinase from *Pyrococcus horikoshii*

MPFVVIITGI PGVGKSTITK LALQRTRAKF KLINFGDLMF EEALKLKLVK HRDEMRKLPL EVQRELQMNA
AKKIAEMAKN YPILLDTHAT IKTPHGYLLG LPYEVIKILN PNFIVIIEAT PSEILGRRLR DLKRDRDVET
EEQIQRHQDL NRAAAITYAM HSNALIKIIE NHEDKGLEEA VNELVKILDL AVKEYA

6 - Adenylate kinase from *Pyrococcus abyssi*

MSFVVIITGI PGVGKSTITR LALQRTKAKF KLINFGDLMF EEAVKAGLVN HRDEMRKLPL EIQRDLQMKV
AKKISEMARQ QPILLDTHAT IKTPHGYLLG LPYEVIKTLN PNFIVIIEAT PSEILGRRLR DLKRDRDVET
EEQIQRHQDL NRAAAIAYAM HSNALIKIIE NHEDKGLEEA VNELVEILDL AVKEYA

7 - Adenylate kinase from *Methanococcus thermolithotrophicus*

MKNKLVVVTG VPGVGGTTIT QKAMEKLSEE GINYKMVNFG TVMFEVAQEE NLVEDRDQMR KLDPDTQKRI
QKLAGRKIAE MVKESPVVVD THSTIKTPKG YLPGLPVWVL NELNPDIIIV VETSGDEILI RRLNDETRNR
DLETTAGIEE HQIMNRAAAM TYGVLTGATV KIIQNKNNLL DYAVEELISV LR

8 - Adenylate kinase from *Methanococcus voltae*

MKNKVVVVTG VPGVGSTTSS QLAMDNLRKE GVNYKMVSFG SVMFEVAKEE NLVSDRDQMR KMDPETQKRI
QKMAGRKIAE MAKESPVAVD THSTVSTPKG YLPGLPSWVL NELNPDLIIV VETTGDEILM RRMSDETRVR
DLDTASTIEQ HQFMNRCAAM SYGVLTGATV KIVQNRNGLL DQAVEELTNV LR

9 - Adenylate kinase from *Methanococcus jannaschii*

MMMMKNKVVV IVGVPGVGST TVTNKAIEEL KKEGIEYKIV NFGTVMFEIA KEEGLVEHRD QLRKLPPEEQ
KRIQKLAGKK IAEMAKEFNI VVDTHSTIKT PKGYLPGLPA WVLEELNPDI IVLVEAENDE ILMRRLKDET
RQRDFESTED IGEHIFMNRC AAMTYAVLTG ATVKIIKNRD FLLDKAVQEL IEVLK

10 - Adenylate kinase from *Methanopyrus kandleri*

MGYVIVATGV PGVGATTVTT EAVKELEGYE HVNYGDVMLE IAKEEGLVEH RDEIRKLPAE KQREIQRLAA
RRIAKMAEEK EGIIVDTHCT IKTPAGYLPG LPIWVLEELQ PDVIVLIEAD PDEIMMRRVK DSEERQRDYD
RAHEIEEHQK MNRMAAMAYA ALTGATVKII ENHDDRLEEA VREFVETVRS L

11 - Adenylate kinase from *Methanotorris igneus*

MKNKVVVVTG VPGVGGTTLT QKTIEKLKEE GIEYKMVNFG TVMFEVAKEE GLVEDRDQMR KLDPDTQKRI
QKLAGRKIAE MAKESNVIVD THSTVKTPKG YLAGLPIWVL EELNPDIIVI VETSSDEILM RRLGDATRNR
DIELTSDIDE HQFMNRCAAM AYGVLTGATV KIIKNRDGLL DKAVEELISV LK

12 - Adenylate kinase from *Pyrobaculum aerophilum*

MKIVIVALPG SGKTTILNFV KQKLPDVKIV NYGDVMLEIA KKRFGIQHRD EMRKKIPVDE YRKVQEEAAE
YIASLTGDVI IDTHASIKIG GGYYPGLPDR IISKLKPDVI LLLEYDPKVI LERRKKDPDR FRDLESEEEI
EMHQQANRYY AFAAANAGES TVHVLNFRGK PESRPFEHAE VAAEYIVNLI LRTRQKS

13 - Adenylate kinase from *Rhodothermus marinus.*

14 - Adenylate kinase from *Thermotoga maritima*

MMAYLVFLGP PGAGKGTYAK RIQEKTGIPH ISTGDIFRDI VKKENDELGK KIKEIMEKGE LVPDELVNEV
VKRRLSEKDC EKGFILDGYP RTVAQAEFLD SFLESQNKQL TAAVLFDVPE DVVVQRLTSR RICPKCGRIY
NMISLPPKED ELCDDCKVKL VQRDDDKEET VRHRYKVYLE KTQPVIDYYG KKGILKRVDG TIGIDNVVAE
VLKIIGWSDK

Example 5

Genetic modification of adenylate kinases to improve stability.

Site-directed mutants were constructed in the AK gene from *P.furiosus*, *P.horikoshii* and *S.acidocaldarius* as shown in Examples 6-8 and SEQ IDs 15-17 respectively, using standard methods known to those familiar with the art. In addition to specific changes identified in each gene, the regions underlined in the *S.acidocaldarius* sequence form the core packing region of the archaeal adenylate kinase trimer structure. Hence amino acid substitutions that disturb the packing of this region are likely to have a major effect in decreasing the thermal and physical stability of the enzyme. Conversely amino aid substitutions that improve the core packing, in particular hydrophobic residues with large side chains, may

stabilise the enzyme to heat or other processes. Therefore in addition to the specific mutations already described a number of "selective" approaches were used with localised gene shuffling of related gene sequences in these regions (essentially as described in Stemmer (1994) *Nature* **370**:389-391 and Crameri *et al* (1996) *Nature Biotech.* **14**:315-319) and random PCR-based mutagenesis using degenerate oligonucleotides or modified nucleotide mixes (e.g. Vartanian *et al* (1996) *Nucleic Acid Res.***24**:2627-2633). A number of these modifications show altered stability when assessed by recombinant expression in *E.coli* and rapid assay of adenylate kinase activity in lysed cells at high temperature.

Example 6

Adenylate kinases from *Pyrococcus furiosus* genetically engineered to provide improved stability (SEQ ID NO. 15).

MPFVVIITGI PGVGKSTITR LALQRTKAKF RLINFGDLMF EEAVKAGLVK HRDEMRKLPL **(K TO E)** IQRELQMKA AKKI **(T TO A)** EMAKE HPILVDTHAT IKTPHGY**(M TO L)** LG LPYEVVKTLN PNFIVIIEAT PSEILGRRLR DLKRDRDVET EEQIQRHQDL NRAAAI**A**YAM HSNALIKIIE NHEDKGLEEA VNELVKILDL AVNEYA

Mutations at one or more or all of the sites indicated modify the thermostability of the enzyme. In addition to the three defined changes highlighted, modification of the alanine at position 157 to another small hydrophobic residue (such as I, L) or larger hydrophobic residue (such as F) increases the thermostability of the recombinant protein. Hence, there are 35 variants possible through combination of modifications at these sites. Modification of amino acid 157 to a polar residue such as the T (as observed at the equivalent position in AdkA of *P.horikoshii*), S Y, D, E, K, R results in a decrease in stability.

Example 7

Adenylate kinases from *Pyrococcus horikoshii* genetically engineered to provide improved stability (SEQ ID NO. 16).

The modification of either or both of the residues shown in bold and underlined increases the thermal stability of the enzyme (3 variants are possible).

MPFVVIITGI PGVGKST ITK LALQRTRAKF KLINFGDLMF EEALKIGLVK HRDEMRKLPL EVQRELQMNA
AKKIAEMAKN YPILLDTHAT IKTPHGYLLG LPYEVIKILN PNFIVIIEAT PSEILGRRLR DLKRDRDVET
EEQIQRHQDL NRAAAI AYAM HSNALIKIIE NHEDKGLEEA VNELVKILDL AVKEYA

Example 8

Adenylate kinase from *Sulfolobus acidocaldarius* genetically engineered to provide improved stability (SEQ ID NO. 17).

The modification of either of the residues shown in bold and underlined increases the thermal stability of the enzyme.

MKIGIVTGIP GVGKSTVLAK VKEILDNQGI NNKIINYGD F MLATALKLGY AKDRDEMRKL SVEKQKKLQI
DAAKGIAEEA RAGGEGYLFI DTHAVIRTPS GY **(A TO M)** PGLPSYV ITEINPS VIF LLEADPKIIL
SRQKRDTTRN RNDYSDESVI L ETINFARYA ATASAVLAGS TVKVIVNVEG DPSIAANEII RSMK

Example 9

An adenylate kinase indicator for the validation of inactivation of TSE

AK-based prion indicators were developed for use with protease inactivation of TSE material at elevated temperature and pH.

Indicator 1

A polycarbonate support was coated with a formulation of purified recombinant *S.acidocaldarius* AK (as described in Example 4). The enzyme was formulated at a concentration of 1mg/ml in the presence of 5% (w/v) sorbitol, 10mg/ml bovine serum albumen (Fraction V; Sigma chemical company) in phosphate buffered saline pH 7.4 (PBS). A volume of 100µl was dried onto the support at 22°C for 1 hour.

Indicator 2

A polystyrene support was coated with 100 microlitres of a formulation containing 1mg/ml *S.acidocaldarius* AK, 1mM tryptophan, 5% (w/v) sorbitol in Tris buffered saline (TBS) pH 7.4 and dried for 24 hours at 4 degrees C.

Protease treatment

An instrument wash bath was set up to operate at 60°C and an alkaline protease formulation added to give between 1.5 and 2 mg/ml of enzyme buffered at pH 12 (as measured at the bath temperature). Suitable anionic detergents may also be included in the formulation if desired. The indicator was incubated for 30 minutes under the conditions described. The indicator was then removed and rinsed once with distilled water. The enzyme activity was then measured as described in Example 1 and luminescence measured using a luminometer.

Standard curves for adenylate kinase enzyme

Standard curves for the AK enzyme was prepared as follows. Serial dilutions of purified *Sulfolobus acidocaldarius* AK from 10 microgrammes/ml to 1fg/ml were prepared in 50mM Tris, 25mM MESNA, pH 7.3. 100 microlitres of enzyme was added to each well of a microtitre plate and 100 microlitres of 135micromolar ADP 15mM MgAc, 1mM EDTA added to each well. Three separate standard curves were prepared for incubation of the assay plate at 30 degrees C, 50 degrees C and 70 degrees C for 20 minutes. Following incubation 30 microlitres of luciferin/luciferase reagent (Biothema) was added and the signal read in a luminometer (Orion, Berthold) and the results shown in Figure 3.

Validation of TSE inactivation

The level of enzyme activity was assessed against the standard curve for the enzyme. For an indicator carrying 100 microgrammes of enzyme with a luminescence value of greater than 1,000,000 Relative Light Units (RLU), as assessed from the standard curve, a 6-log inactivation in signal correlates with a luminescence value of less than 4000 RLU and an 8-log inactivation correlates with a luminescence value of less than 500 RLU.

Under the protease digestion conditions described above (2mg/ml alkaline protease designated MC3 in a buffer formulation at pH12 and digestion for 30 minutes at 60 degrees C) we demonstrated a reduction of approximately 8-logs in the levels of infectivity of the BSE-301V strain bioassayed in VM mice. Under the same conditions an AK indicator formulated as described above showed a reduction in RLU from in excess of 1,000,000 (untreated) to less than 500 RLU. The correlation between the

8-log loss of infectivity and the >8-log reduction in the corresponding levels of indicator activity allowed a demonstration that the protease treatment process had run effectively.

Use of validated procedure

A set of surgical instruments used in routine surgery or routine neurosurgery for a patient not known to be incubating any form of CJD is returned to a hospital sterile services unit following use. The instruments are prepared for routine cleaning in a washer/disinfector set to operate at 60°C for 30 minutes with a formulation of alkaline protease MC3. One or more AK indicators as described above are included in the processing bath. After treatment, and before instruments are sent on for normal autoclave sterilisation, the indicator is removed and a rapid assay carried out to confirm that the process is effective. An assay result that demonstrates a 6- or 8-log loss of activity, as defined by the parameters of the process, is required before any instruments can be processed further. Following a successful process, instruments may be prepared for other sterilisation procedures such as autoclaving as required.

Example 10

Expression of acetate and pyruvate kinases

Following the methods of Example 4, we expressed acetate and pyruvate kinases:-

18 - Acetate kinase from *Thermatoga maritima*

MRVLVINSGS SSIKYQLIEM EGEKVLCKGI AERIGIEGSR LVHRVGDEKH VIERELPDHE EALKLILNTL
VDEKLGVIKD LKEIDAVGHR VVHGGERFKE SVLVDEEVLK AIEEVSPLAP LHNPANLMGI KAAMKLLPGV
PNVAVFDTAF HQTIPQKAYL YAIPYEYYEK YKIRRYGFHG TSHRYVSKRA AEILGKKLEE LKIITCHIGN
GASVAAVKYG KCVDTSMGFT PLEGLVMGTR SGDLDPAIPF FIMEKEGISP QEMYDILNKK SGVYGLSKGF
SSDMRDIEEA ALKGDEWCKL VLEIYDYRIA KYIGAYAAAM NGVDAIVFTA GVGENSPITR EDVCSYLEFL
GVKLDKQKNE ETIRGKEGII STPDSRVKVL VVPTNEELMI ARDTKEIVEK IGR

19 - Pyruvate kinase from *Pyrococcus horikoshii*

MRRMKLPSHK TKIVATIGPA TNSKKMIKKL IEAGMNVARI NFSHGTFEEH AKIIEMVREQ SQKLDRRVAI
LADLPGLKIR VGEIKGGYVE LERGEKVTLT TKDIEGDETT IPVEYKDFPK LVSKGDVIYL SDGYIVLRVE

DVKENEVEAV VISGGKLFSR KGINIPKAYL PVEAITPRDI EIMKFAIEHG VDAIGLSFVG NVYDVLKAKS
FLERNGAGDT FVIAKIERPD AVRNFNEILN AADGIMIARG DLGVEMPIEQ LPILQKRLIR KANMEGKPVI
TATQMLVSMT MEKVPTRAEV TDVANAILDG TDAVMLSEET AVGKFPIEAV EMMARIAKVT
EEYRESFGIT RMREFLEGTK RGTIKEAITR SIIDAICTIG IKFILTPTKT GRTARLISRF KPKQWILAFS
TREKVCNNLM FSYGVYPFCM EEGFNENDIV RLIKGLGLVG SDDIVLMTEG KPIEKTVGTN SIKIFQIA

Example 11

Thermostable adenylate kinase as an indicator for proteolytic inactivation of TSE agent.

A 20mg/ml stock of alkaline protease was diluted using a solution of 10µg/ml recombinant *S.acidocaldarius* AK in buffered 0.2M KCl pH 12, to generate a range of protease concentrations from 2 mg/ml down to 0.001µg/ml. A volume of 100µl of material was added to a polystyrene thermocycler plate and incubated for 10 minutes at 60°C to allow digestion of the AK by the alkaline protease. The solution was neutralised by addition of 10µl of 10 x Phosphate buffer pH 7. 100µl/well of 135µM ADP in 15mM MgAc, 1mM EDTA buffer was added. The wells were then incubated for 20mins at 70°C in a thermocycler. 30µl/well of Luciferin/Luciferase (ATP) reagent (Biothema) was added and the wells read on the luminometer immediately. The results of the assay are shown in Figure 2.

These results show the remarkable stability of the AK enzyme to digestion by alkaline protease under conditions identified as being useful for the decontamination of surgical instruments. To relate the reduction observed following digestion with alkaline protease to the levels of activity remaining after treatment a standard curve such as that shown in Figure 3 is used. In turn this is related to the degree of reduction in the levels of infectivity using a titration curve of TSE. For use as an indicator the assay is designed such that the standard curve for enzyme activity is clearly marked with a limit value correlating with an acceptable level of decontamination by the process.

	Organism	Domain	Growth	T_{opt}	pH_{opt}
1	Aeropyrum pernix	Archaeon	Aerobe	95°C	7.0
2	Alicyclobacillus acidocaldarius	Bacterium	Aerobe	65°C	3.5
3	Aquifex pyrophilus	Bacterium	Microaerophileeberophile	85°C	6.5
4	Bacillus caldotenax BT1	Bacterium	Aerobe	65°C	7.0
5	Bacillus species PS3	Bacterium	Aerobe	65°C	7.0
6	Bacillus stearothermophilus 11057	Bacterium	Aerobe	65°C	7.0
7	Bacillus stearothermophilus 12001	Bacterium	Aerobe	65°C	7.0
8	Bacillus thermocatenulatus	Bacterium	Aerobe	65°C	7.0
9	Clostridium stercocorarium	Bacterium	Anaerobe	55°C	7.0
10	Meiothermus ruber	Bacterium	Aerobe	60°C	6.5
11	Pyrococcus furiosus	Archaeon	Anaerobe	95°C	7.5
12	Pyrococcus horikoshii	Archaeon	Anaerobe	95°C	7.0
13	Pyrococcus woesei	Archaeon	Anaerobe	95°C	7.0
14	Rhodothermus marinus	Bacterium	Aerobe	70°C	6.5
15	Sulfolobus acidocaldarius 98-3	Archaeon	Aerobe	75°C	2.5
16	Sulfolobus shibatae B21	Archaeon	Aerobe	75°C	2.5
17	Sulfolobus solfataricus P2	Archaeon	Aerobe	75°C	2.5
18	Thermoanaerobacter ethanolicus	Bacterium	Anaerobe	65°C	6.0
19	Thermoanaerobacter thermosulfurogenes	Bacterium	Anaerobe	65°C	6.5
20	Thermobrachium celere	Bacterium	Anaerobe	60°C	7.0
21	Thermococcus litoralis	Archaeon	Anaerobe	85°C	6.5
22	Thermus aquaticus YT1	Bacterium	Aerobe	70°C	8.0
23	Thermus caldophilus GK24	Bacterium	Aerobe	70°C	8.0
24	Thermus thermophilus HB8	Bacterium	Aerobe	70°C	8.0

Table 1

List of thermophilic organisms cultured to produce biomass for isolation of thermostable AKs.

CLAIMS

1. A composition, comprising:-

(i) a kinase, and

(ii) a solid support,

wherein the kinase is immobilised in or is immobilised on the solid support.

2. A composition according to Claim 1 wherein the kinase is thermostable.

3. A composition according to Claim 2 wherein the kinase retains at least 95% of its activity after exposure to 80°C for 10 minutes.

4. A composition according to any of Claims 1-3 wherein the kinase is adenylate kinase, acetate kinase or pyruvate kinase.

5. A composition according to any of Claims 1-4 wherein the kinase catalyses formation of ATP from a substrate comprising ADP.

6. A composition according to any of Claims 1-5 wherein the solid support is a matrix and the kinase is disbursed within the matrix.

7. A composition according to Claim 6 wherein the support comprises a polymer matrix.

8. A composition according to any of Claims 1-7 wherein the support is an indicator strip, a dip stick or a bead.

9. A composition according to any of Claims 1-8 comprising an agent to stabilise the kinase.

10. A composition according to Claim 9 wherein the stabilising agent is selected from metal ions, sugars, sugar alcohols, gel-forming agents etc.

11. A composition according to any of Claims 1-10, further comprising means to attach the composition to a surface.

12. A composition according to Claim 11 comprising a projection, recess or aperture for attachment of the composition to a surface by means of a screw, nut and bolt or clamp.

13. A kit for determining whether an infectious agent has been inactivated, comprising:-

(i) a composition according to any of Claims 1-12, and

(ii) substrate for the kinase.

14. A kit according to Claim 13, further comprising means for detecting ATP.

15. A kit according to Claim 14, further comprising luciferin/luciferase.

16. A kit according to any of Claims 13-15 further comprising a luminometer.

17. A kit according to any of Claims 13-16 further comprising a look-up table correlating kinase inactivation with infectious agent inactivation.

18. A kit according to any of Claims 13-17 for monitoring TSE inactivation.

19. A portable kit according to any of Claims 13-18.

20. A method of determining the extent of inactivation of an infectious agent, comprising:-

(i) obtaining a sample containing the infectious agent and a known amount of a kinase;

(ii) treating the sample to inactivate the infectious agent;

(iii) measuring residual kinase; and

(iv) thereby determining residual infectious agent.

21. A method according to Claim 20 wherein the extent of inactivation of the infectious agent correlates with the extent of inactivation of the kinase.

22. A method according to Claim 20 or 21 comprising obtaining a sample believed to contain the infectious agent and adding a known amount of kinase.

23. A method according to any of Claims 20-22, comprising measuring kinase activity prior to treating the sample and after treating the sample.

24. A method according to any of Claims 20-23, comprising treating the sample at 80°C for at least 10 minutes prior to measuring residual kinase.

25. A method according to any of Claims 20-24 wherein the treatment comprises one or more of high pH, high temperature and high pressure.

26. A method according to any of Claims 20-25 wherein the treatment comprises exposing the sample to a protease.

27. A method according to Claim 26, wherein the treatment comprises exposing the sample to a thermostable protease at a temperature of 60°C or above.

28. A method according to Claim 27 wherein the treatment comprises exposing the sample to the protease at a pH of 9 or above.

29. A method according to any of Claims 20-28 wherein measuring residual kinase comprises adding a substrate comprising ADP to the residual kinase and measuring formation of ATP.

30. A method of correlating inactivation of an infectious agent by a treatment with inactivation of an indicator by the treatment, comprising:-

(i) obtaining a sample containing a known amount of the infectious agent and a known amount of the indicator;

(ii) carrying out the treatment on the sample;

(iii) measuring residual infectious agent; and

(iv) measuring residual indicator.

31. A method according to Claim 30 wherein the indicator is a kinase.

32. A method according to Claim 31 wherein the kinase is adenylate kinase, acetate kinase or pyruvate kinase.

33. A method according to any of Claims 30-32 wherein the indicator is detectable after a reduction in its activity of 8 logs or more.

34. A method according to any of Claims 30-33 wherein the infectious agent is a transmissible spongiform encephalopathy.

35. A method according to any of Claims 30-34 wherein the treatment comprises high temperature and/or high pH.

36. A method according to any of Claims 30-35 where the treatment comprises exposing the sample to a protease.

37. A method of treating a sample containing an infectious agent, comprising:-

adding a biological indicator to the sample;

treating the sample;

assaying for residual activity of the biological indicator; and

continuing the treatment until the biological indicator activity indicates a reduction in activity of transmissible spongiform encephalopathy of 8 logs or greater.

38. A method according to Claim 37 wherein the biological indicator comprises a kinase.

39. A method according to Claim 37 or 38 wherein the treatment comprises exposing the sample to a thermostable protease at high temperature and high pH.

40. Use of a thermostable kinase in manufacture of a biological indicator.

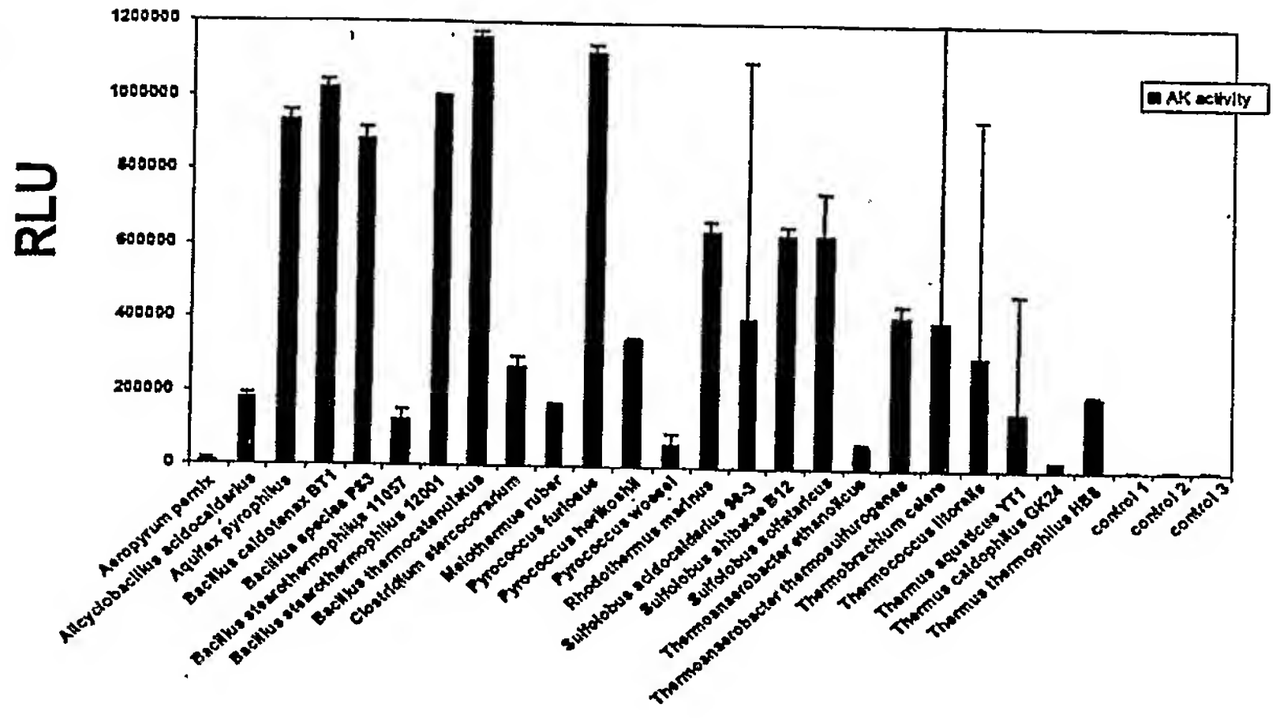
41. Use according to Claim 40 wherein the biological indicator is for validating inactivation of infectious agents.

BIOLOGICAL INDICATOR

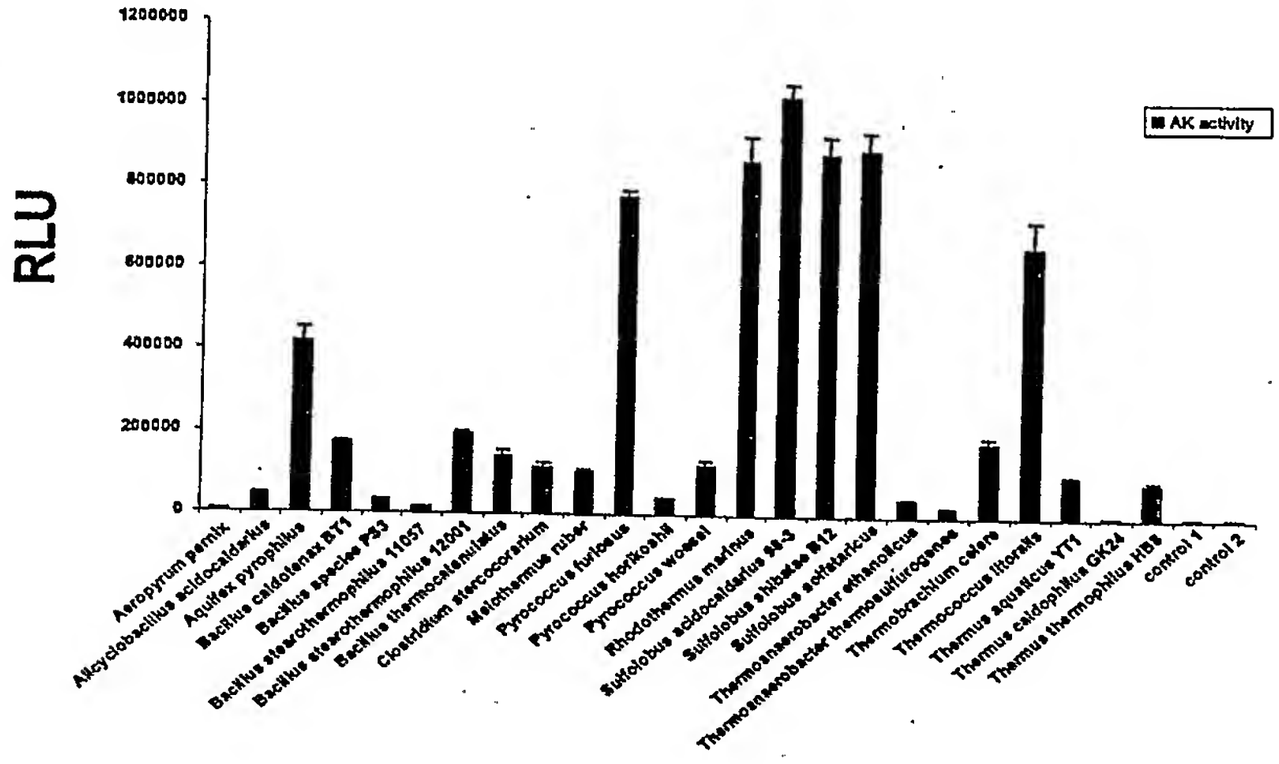
ABSTRACT

A kinase immobilised on a solid support is used in a biological indicator for validation of processes designed to inactivate infectious agents.

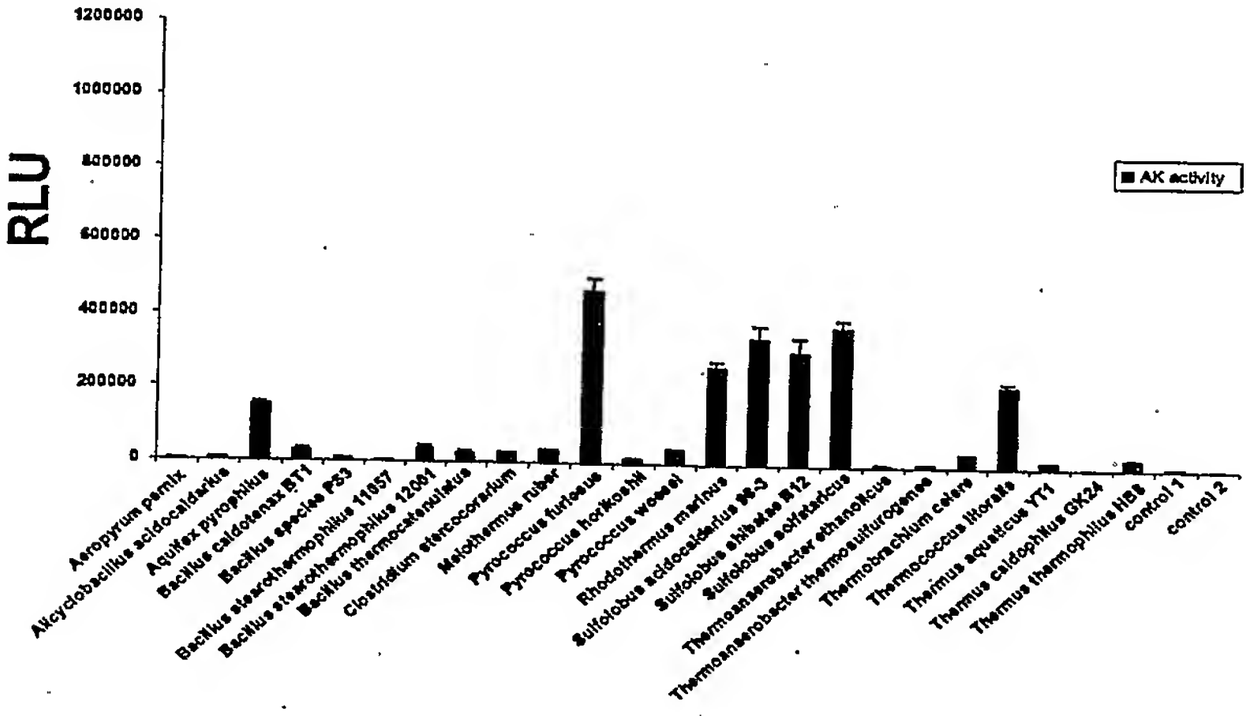
A



B



C



RLU = Relative light units

Fig. 1

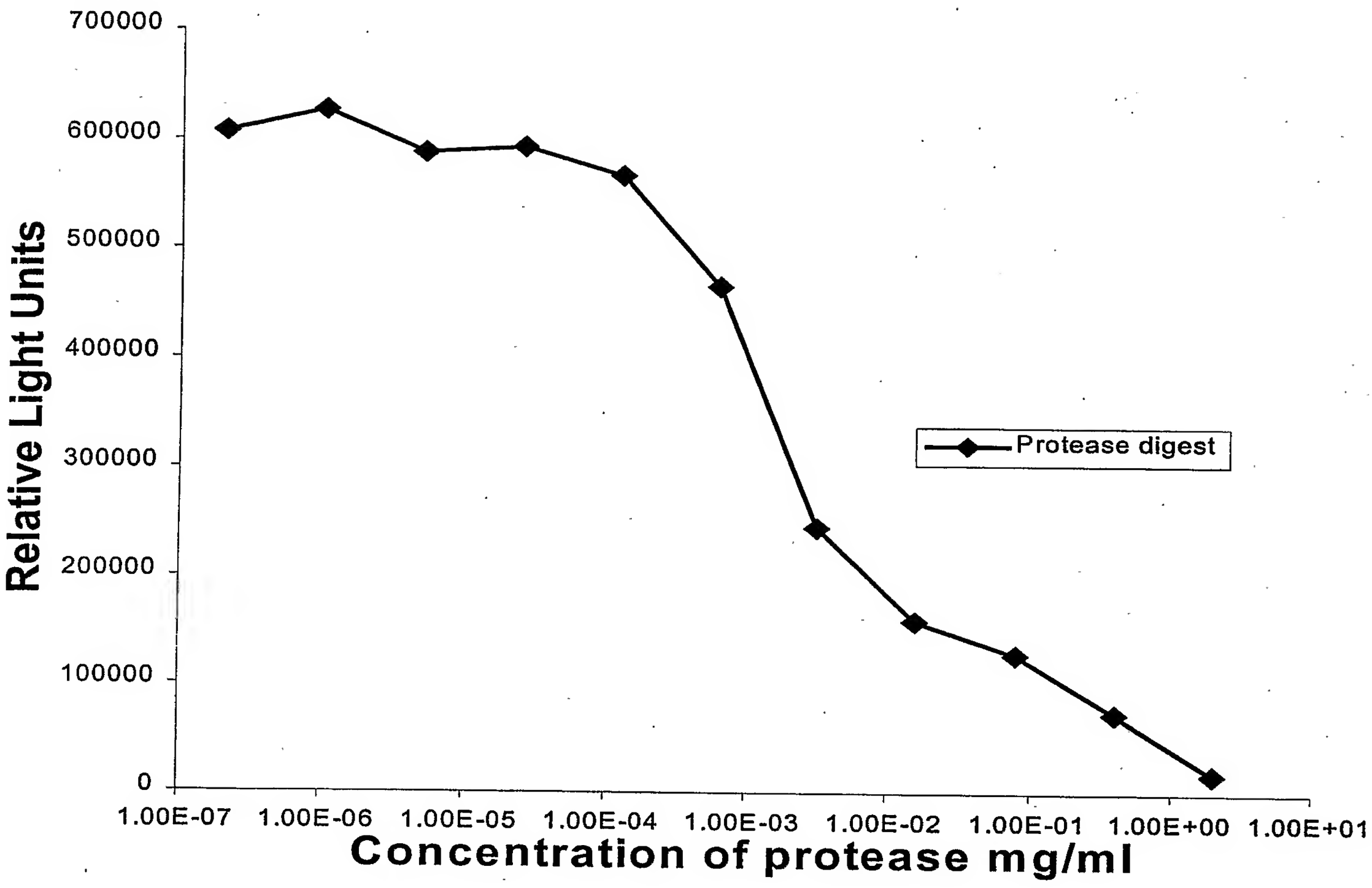

700000
600000
500000
400000
300000
200000
100000
0
Relative Light Units
1.00E-07
1.00E-06
1.00E-05
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
1.00E+01
Concentration of protease mg/ml
Protease digest

Fig. 2

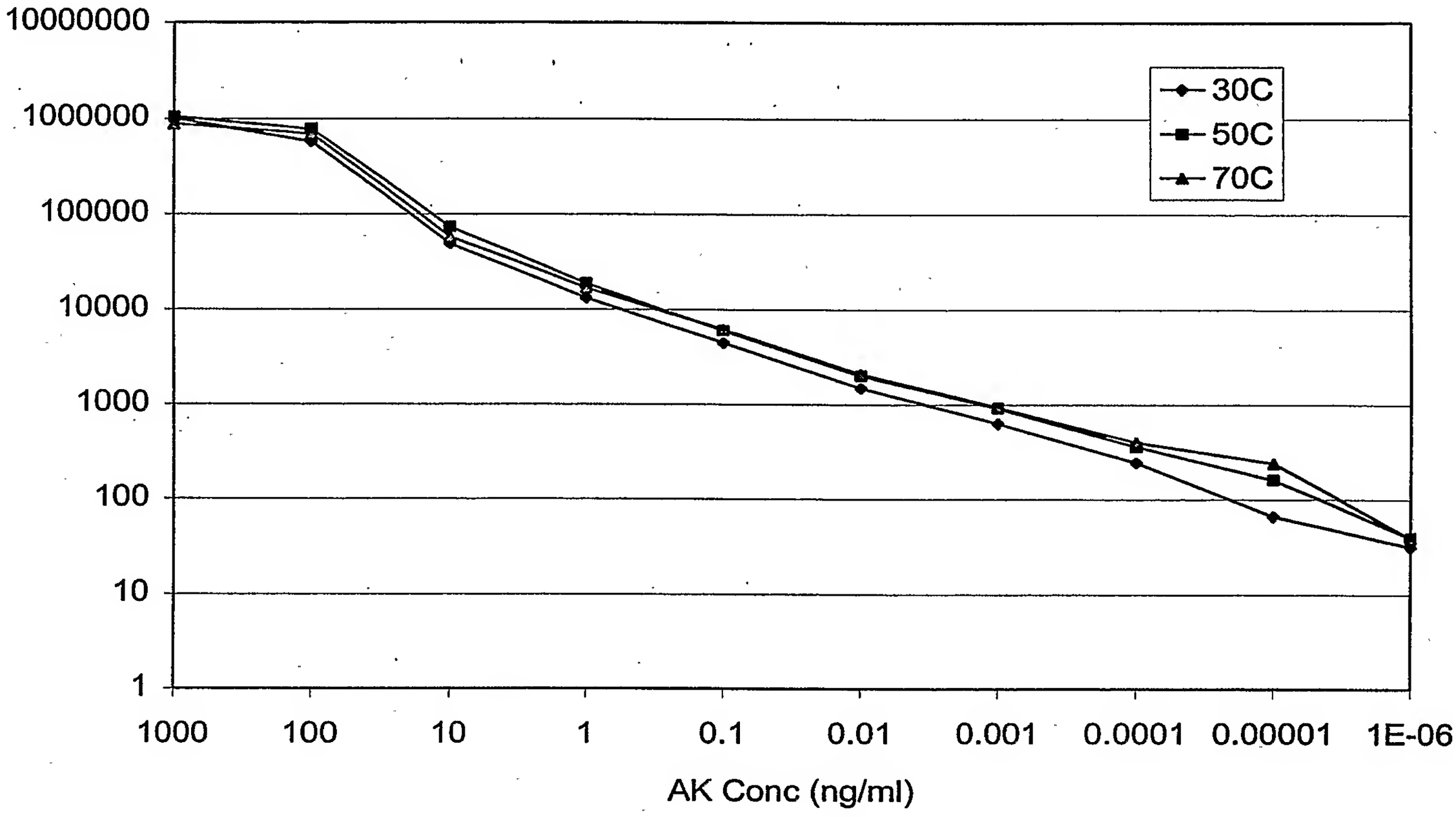

Dynamic range of adenylate kinase indicator with varying temperature
10000000
1000000
100000
10000
1000
100
10
1
30C
50C
70C
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
1E-06
AK Conc (ng/ml)

Fig. 3